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Keywords
Ovary, Obesity, 7, 12-dimethylbenz[a]anthracene, Gap junction communication, Connexin

Disciplines
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Impact of obesity on 7,12-dimethylbenz[a]anthracene-induced altered ovarian connexin gap junction proteins in female mice

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Abstract

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Keywords

Ovary; Obesity; 7, 12-dimethylbenz[a]anthracene; Gap junction communication; Connexin

Introduction

The ovary is the major female reproductive organ composed of follicles at different developmental stages from primordial to antral. Follicles contain a single oocyte, arrested in the diplotene stage of meiosis, which are surrounded by single to multiple layers of

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granulosa cells dependent on the stage of development. Granulosa cells are the somatic follicular cells and their functions include production of sex steroid hormones and growth factors which are essential for fertile reproductive life of women. Granulosa cell death by apoptosis is reportedly involved in the process of follicular atresia in the mammalian ovary (Tilly, 1997; Jiang *et al.*, 2003).

Connexins (CX) are a family of transmembrane proteins that connect to form gap junctions; channels which allow direct exchange of ions and small molecules between adjacent cells and which are involved in cell proliferation, differentiation, cell survival, oocyte maturation, meiotic resumption and death (Gershon *et al.*, 2008; Conti *et al.*, 2012; Kar *et al.*, 2012). Eight CX proteins are expressed in ovaries encoded by the *Cx26*, *Cx30.3*, *Cx32*, *Cx37*, *Cx40*, *Cx43*, *Cx45* and *Cx57* genes (Grazul-Bilska *et al.*, 1997; Kidder and Mhawi, 2002). The most abundant CX protein expressed in mouse ovaries are CX37, CX43 and CX45 (Simon *et al.*, 1997; Ackert *et al.*, 2001; Wright *et al.*, 2001). CX37 is present between the oocyte and granulosa cell (Simon *et al.*, 1997) while CX43 (Valdimarsson *et al.*, 1993; Gittens, 2003) and CX45 (Okuma *et al.*, 1996; Alcoléa *et al.*, 1999; Wright *et al.*, 2001) co-localize between granulosa cells. CX proteins are expressed in a temporal pattern during follicular development and maturation in mouse ovaries (Wright *et al.*, 2001), and defects in oocyte and follicular development have been identified in CX37 deficient mice in which heterologous oocyte-granulosa cell gap junctions were under developed (Carabatsos *et al.*, 2000). *Cx37*-null mouse oocytes suffer growth retardation and do not survive to become meiotically competent (Carabatsos *et al.*, 2000) due to lack of nutrient intake (Eppig, 1991). CX43 protein expression was also reduced in ovarian granulosa cells during follicular atresia in pigs and swamp buffaloes (Cheng *et al.*, 2005; Feranil *et al.*, 2005). Folliculogenesis and oocyte growth are impaired past the primary stage in *Cx43*-deficient mice (Juneja *et al.*, 1999; Ackert *et al.*, 2001). These studies indicate that CX proteins are important for completion of oocyte growth, acquisition of cytoplasmic meiotic competence and follicular survivability.

CX proteins are targeted by chemicals including retinoids, carotinoids, chemotherapeutic agents (Trosko and Ruch, 2002); Upham and Trosko, 2009; King and Bertram, 2005), cigarette components (Upham *et al.*, 1996; Tai *et al.*, 2007; Upham *et al.*, 2008; Upham and Trosko, 2009); McKarns *et al.*, 2000) and polycyclic aromatic hydrocarbons including 7,12-dimethylbenz[a]anthracene (DMBA) (Sharovskaya *et al.*, 2006; Ganesan and Keating, 2014; (Trosko, 1989). DMBA, liberated from burning of organic matter incineration, destroys all type of follicles in the ovaries of exposed mice and rats, leading to ovarian failure (Gelboin, 1980; Mattison and Schulman, 1980). Cigarette smoke causes premature menopause onset in female smokers compared to their age matched non-smoking counterparts (Mattison *et al.*, 1983; Alcoléa *et al.*, 1999; Harlow and Signorello, 2000) and the offspring of female smokers have decreased numbers of oocytes, potentially leading to infertility (Jurisicova *et al.*, 2007). DMBA destroys follicles by inducing apoptosis through increased expression of pro-apoptotic BAX and activation of the executioner protein caspase 3 (Tilly *et al.*, 1991; Tsai-Turton *et al.*, 2007). Additionally, DMBA altered CX protein expression when neonatal cultured rat ovaries were exposed *in vitro* (Ganesan and Keating, 2014).

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Approximately one-third of adults in the USA are obese (Flegal Km *et al.*, 2010; Meeker *et al.*, 2010), and negative female phenotypic associations include polycystic ovarian syndrome, menstrual disorders, intrauterine fetal death and infertility (Haslam and James; Cardozo *et al.*, 2012). Obesity also detrimentally affects pregnancy rates in natural and assisted conception potentially by reducing oocyte quality (Wu *et al.*, 2011). Primordial and small primary follicle number were reduced in ovaries from obese mice with a concomitant increase in the number of secondary and pre-ovulatory follicles relative to lean mouse ovaries (Nteeba *et al.*, 2014a). Additionally, ovaries from mice fed a high fat diet showed increased accumulation of endoplasmic reticulum stress, decreased mitochondrial activity and increased apoptosis of cumulus oocyte complexes and ovarian cells (Wu *et al.*, 2010). Interestingly, a high fat diet also reduced cardiovascular *Cx* expression in female rats resulting in increased risk of ventricular arrhythmia (Aubin *et al.*, 2010). Taken together, both DMBA and obesity have separately been shown to affect *Cx* gene mRNA and protein levels in non-ovarian tissues, thus this study investigated their impact on ovarian *Cx* mRNA and protein levels using a mouse model of progressive obesity.

Methods and Materials

Reagents

DMBA (CAS # 57-97-6), sesame oil (CAS # 8008-74-0), 2-β-mercaptoethanol, 30% acrylamide/0.8% bisacrylamide, ammonium persulphate, glycerol, N′N′N′N′-Tetramethylethylenediamine (TEMED), Tris base, Tris HCL, Sodium chloride, Tween-20 were purchased from Sigma-Aldrich Inc. (St Louis, MO). RNeasy Mini kit, QIA shredder kit, RNeasy Min Elute kit, and Quantitect TM SYBR Green PCR kit were purchased from Qiagen Inc (Valencia, CA). All primers were purchased from the Iowa State University DNA facility. All primary antibodies were purchased from Abcam (Cambridge, MA). RNA later was obtained from Ambion Inc. (Austin, TX). Goat anti-rabbit secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Ponceau S was from Fisher Scientific. ECL plus chemical luminescence detection kit was obtained from GE Healthcare, Amersham (Buckinghamshire, UK).

Animals

The ovarian tissues utilized in this study were obtained as part of a larger study by our group (Nteeba *et al.*, 2014a; Nteeba *et al.*, 2014b). Briefly, four week old female wild type normal non-agouti (a/a; designated lean) and agouti lethal yellow (KK.Cg-Ay/J; designated obese) were purchased from Jackson laboratories (Bar Harbor, ME 002468). All animals were housed in cages under a 12 h light/dark photoperiod with the temperature between 70–73°F and humidity approximately 20–30%. The animals were provided with a standard diet (Teklad 2014 global 14% protein rodent maintenance diet) with *ad libitum* access to food and water until 6, 12, 18 or 24 wks of age (n = 5 per strain, per time point). Also, a subset of 18 wk old mice were dosed with sesame oil (vehicle control; n = 5 lean; n = 5 obese) or DMBA (1mg/kg; intraperitoneal injection; n = 5 lean; n = 5 obese) for 14 days and ovaries collected 3 days after the end of exposure. At the end of DMBA dosing and tissue collection, animals were 20.5 wks of age. This DMBA dose was chosen based on the literature to induce approximately 50% primordial follicle loss (Borman *et al.*, 2000). Long

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term phenotypic impact observations of DMBA on fertility were outside the timeline of this study. All procedures were approved by the Iowa State University Animal Care and Use Committee.

Tissue collection

Mice were euthanized in their pro-estrus phase of cyclicity by CO_2 asphyxiation. Ovaries were collected and cleaned. One ovary was stored in RNA*later* at -80°C for RNA and protein analyses with the contralateral ovary was fixed in 4% paraformaldehyde for immunostaining. The ovary stored at -80°C was powdered and evenly divided for RNA and protein isolation. As a note, one ovary from an obese DMBA-treated female could not be localized therefore the final number in this group was n = 4, with n = 5 for all other treatments.

RNA isolation and quantitative RT-PCR

RNA was isolated (n = 4–5) using an RNeasy Mini kit (Qiagen) and the concentration determined using an ND-1000 Spectrophotometer (λ = 260/280nm; NanoDrop technologies, Inc., Wilmington, DE) (n=3). Total RNA (200 ng) was reverse transcribed to cDNA utilizing the Superscript III One-Step RT-PCR (Qiagen). Three randomly chosen cDNA samples per treatment were diluted (1:20) in RNase-free water and amplified in triplicate on an Eppendorf PCR Master cycler using a Quantitect SYBR Green PCR kit (Qiagen). Primers for *Cx37*, *Cx43* and *Cx45* and *Gapdh* were designed by Primer 3 Input Version (0.4.0) (Table 1). The regular cycling program consisted of a 15 min hold at 95°C and 45 cycles of denaturing at 95°C for 15 s, annealing at 58°C for 15 s, and extension at 72°C for 20 s at which point data were acquired. There was no difference in *Gapdh* mRNA expression between treatments, thus each sample was normalized to *Gapdh* before quantification. Quantification of fold-change in gene expression was performed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Pfaffl, 2001).

Protein isolation and western blotting

Protein was isolated from whole ovaries (n = 4–5) by homogenization in tissue lysis buffer containing protease and phosphatase inhibitors as previously described (Ganesan and Keating, 2014). Briefly, homogenized samples were placed on ice for 30 min, followed by two rounds of centrifugation at 10,000 rpm for 15 min and protein concentration was measured using a standard BCA protocol. SDS-PAGE was used to separate protein homogenates from three randomly chosen samples per time point or treatment which were then transferred to a nitrocellulose membrane. Membranes were blocked for 1 hour in 5 % milk in Tris-buffered saline containing Tween 20, followed by incubation in anti-rabbit CX37, CX43 and CX45 primary antibodies (1:100) for 36 h at 4°C. Following three washes in TTBS (1X), membranes were incubated with species-specific secondary antibodies (1:3000) for 1 h at room temperature. Membranes were washed 3X in TTBS and incubated in enhanced chemiluminescence detection substrate (ECL plus) for 5 min followed by X-ray film exposure. Densitometry of the appropriate bands was performed using ImageJ software (NCBI). Equal protein loading was confirmed by Ponceau S staining of membranes and protein level was normalized to Ponceau S densitometry values.

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Immunofluorescence staining

Ovaries (n = 4–5) were serially sectioned (5 μM thickness) and every 10th section was mounted. Slides from three randomly chosen ovaries were deparaffinized in xylene and rehydrated with subsequent washes in ethanol. Antigen retrieval was carried out by microwaving sections for 7 min in sodium citrate buffer (1M, pH 6.1). Sections were then blocked in 5% BSA for 1 h at room temperature. Sections were incubated with primary antibody directed against CX37 (1:200), CX43 (1:200) or CX45 (1:100) overnight at 4°C. After washing in 1% PBS, sections were incubated with the appropriate goat anti-rabbit IgG-FITC secondary antibody for 1 h. Slides were then counterstained with 4–6-diamidino-2-phenylindole (DAPI) nuclear stain for 5 min. Images were captured using a Leica fluorescent microscope and protein expression analyzed using ImageJ software (NCBI). Immunofluorescence staining for CX37, CX43 or CX45 was quantified within the entire follicle; 10 antral follicles per ovary and 3 ovaries (randomly chosen) were used.

Statistical analysis

Sufficient numbers of animals and repetitions within analyses were utilized for statistical analysis. Raw data were analyzed by unpaired t-test using Graphpad Prism 5.04 software. Values are expressed as fold change ± SE for mRNA expression and raw data mean ± SE for protein results, n=3. Different letters or asterisk (*) indicate statistical significance at $P < 0.05$.

Results

Effect of progressive obesity on *Cx37* mRNA and protein abundance

In lean mice, *Cx37* mRNA levels were increased ($P < 0.05$) at 24 wks compared to earlier time points (Figure 1A). This increase was absent in obese ovaries (Figure 1B). Comparison of both strains demonstrated lower ($P < 0.05$) ovarian *Cx37* mRNA abundance in the obese mice at 24 wks of age (Figure 1C). In lean mice, ovarian CX37 protein level was decreased ($P < 0.05$) at 18 and 24 wks, relative to 6 and 12 wks of age, with this decline being greater ($P < 0.05$) at 24 wks than 18 wks (Figure 1D). In obese mice, CX37 protein also progressively decreased over time (Figure 1E). Ovarian CX37 protein was higher ($P < 0.05$) in obese mice at 12 wks and lower ($P < 0.05$) at 24 wks, relative to lean counterparts (Figure 1F,G).

Progressive obesity impact on *Cx43* mRNA and protein level

Temporally increased ($P < 0.05$) *Cx43* mRNA was observed in both lean and obese mice (Figure 2A,B). Increased ($P < 0.05$) *Cx43* mRNA was evident at 12 wks due to obesity, which was decreased ($P < 0.05$) by 18 and 24 wks of age (Figure 2C). Ovarian CX43 protein levels were slightly increased ($P < 0.05$) temporally in lean mice (Figure 2D) but decreased ($P < 0.05$) over time in obese mice (Figure 2E). Total CX43 was lower ($P < 0.05$) in obese relative to lean ovaries at 18 and 24 wks (Figure 2F,G).

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Effect of obesity on ovarian *Cx45* mRNA and protein abundance

A dramatic decrease ($P < 0.05$) in *Cx45* mRNA over time in both lean and obese ovaries was noted (Figures 3A,B). *Cx45* mRNA abundance was greater ($P < 0.05$) in obese relative to lean ovaries after 12 wks; however this effect was reversed ($P < 0.05$) by 18 and 24 wks of age (Figure 3C). CX45 protein level was also decreased over time in both lean and obese ovaries (Figure 3D,E). In a similar manner to *Cx45* mRNA, protein levels were greater ($P < 0.05$) in ovaries from obese mice at 12 wks of age, and a reduction ($P < 0.05$) observed by 18 and 24 wks of age (Figure 3F,G).

DMBA effects on *Cx37* mRNA and protein level

Basal levels of *Cx37* mRNA were decreased ($P < 0.05$) in obese compared to lean ovaries. DMBA exposure did not impact *Cx37* mRNA abundance (Figure 4A). CX37 total protein levels were not altered by obesity. DMBA did not impact CX37 total protein abundance in lean mice but decreased ($P < 0.05$) CX37 in ovaries from obese mice (Figure 4B,C).

Impact of DMBA exposure on ovarian *Cx43* mRNA and protein

Basal levels of *Cx43* mRNA were not affected by obesity, however, DMBA exposure reduced ($P < 0.05$) *Cx43* levels with an additive effect of obesity on the DMBA-induced reduction (Figure 5A). Obesity did not impact CX43 total protein level, but CX43 protein was decreased ($P < 0.05$) by DMBA exposure with lack of any additive effect of obesity (Figure 5B,C).

Consequence of DMBA exposure on *Cx45* mRNA and protein expression

Obesity reduced ($P < 0.05$) *Cx45* mRNA abundance (Figure 6A), but there was no impact of DMBA exposure thereon. CX45 protein abundance was decreased ($P < 0.05$) by obesity. Levels of CX45 protein were unaltered by DMBA exposure in lean mice, but in the obese females, ovarian CX45 protein was increased ($P < 0.05$; Figure B, C).

Localization and quantification of obesity and DMBA effects on CX proteins

CX37 was localized to the oocyte cytoplasm of the oocyte and between the granulosa cells (Figure 7A–D). CX37 protein basal levels were lower ($P < 0.05$) in obese relative to lean ovaries, but there was no impact of DMBA exposure thereon (Figure 7M). CX43 was localized between granulosa cells (Figure 7E–H). Both obesity and DMBA exposure reduced ($P < 0.05$) CX43 protein levels but no interaction was evident (Figure 7N). CX45 protein was localized between granulosa cells (Figure 7I–L) and was lower ($P < 0.05$) in obese relative to lean ovaries, but no statistical impact of DMBA on CX45 was observed (Figure 7O).

Discussion

Our previous work has demonstrated that the level of microsomal epoxide hydrolase (mEH), an enzyme required for ovarian DMBA bioactivation (Keating *et al*., 2008; Igawa *et al*., 2009), is higher in ovaries of obese mice, potentially predisposing them to increased DMBA-induced ovotoxicity (Nteeba *et al.*, 2014a). Also, obese, DMBA-treated mice had

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elevated levels of a marker of double stranded DNA damage (γH2AX) as well as a blunted ovarian DNA damage repair response (Ganesan *et al*., in press). High fat diet fed mice have increased levels of apoptosis in cumulus oocyte complexes and granulosa cells (Wu *et al.*, 2010) and reduced cardiac *Cx* expression in female rats (Aubin *et al.*, 2010), potentially supporting that obesity alters CX protein abundance. Additionally, DMBA altered *Cx* gene expression in neonatal ovaries (Ganesan and Keating, 2014), however, the effect of DMBA on *Cx* gene expression in adult mouse ovaries has not yet been studied. Thus, the impacts of both DMBA exposure and progressive obesity on ovarian *Cx* mRNA and protein abundance were investigated in ovaries from adult mice.

In lean mice, ovarian *Cx37* mRNA level was increased at 24 wks however total protein decreased after 18 wks potentially indicating that CX37 protein levels decline with ovarian aging. Ovaries from obese mice had lower levels of *Cx37* mRNA and protein after 24 wks compared to lean ovaries, but in a similar manner to the lean mice, *Cx37* mRNA and protein declined with aging in obese females. CX37 is essential for follicular development, ovulation as well as luteal tissue growth, differentiation, and regression (Simon *et al.*, 1997). *Cx37* mRNA was previously shown to be reduced in the mesenteric arteries of 25 wk old insulin resistant obese compared to lean littermate control rats (Young *et al.*, 2008). These results indicate a decline in CX37 levels with ovarian aging.

CX37 protein was localized in the cytoplasm of oocyte (oolemma) and in the granulosa cells of large follicles, consistent with our previous study (Ganesan and Keating, 2014) and others who showed that CX37 is present on the oocyte surface of pre-antral follicles and between the granulosa cells of large antral follicles in mouse ovaries (Wright *et al.*, 2001; Teilmann, 2005; Simon *et al.*, 2006). Shrunken, misshapen oocytes were noted in both the obese as well as DMBA-exposed ovaries, thus analysis was confined to the entire follicular structure. CX37 antral follicle protein staining intensity was reduced in both the obese control and DMBA-treated ovaries compared to lean ovaries, in agreement with our previous work demonstrating that CX37 protein was decreased in cultured neonatal rat ovaries after DMBA exposure (Ganesan and Keating, 2014) as well as with the data from western blotting in the current study. Interestingly, obesity had a greater impact on DMBA-induced decreased CX37, suggesting DMBA may accelerate the decrease in CX37 protein levels during obesity, further contributing to ovotoxicity. Loss of organized CX37 localization around the oocyte perimeter is an early sign of follicular atresia (Teilmann, 2005), and suggests that intact cellular communication between the oocyte and the somatic cells is mandatory for follicular health. Reduced CX37 has been shown in denuded oocytes of diabetic mice compared to non-diabetic mice (Ratchford *et al.*, 2008). Our results indicate that CX37 protein levels are reduced by aging, progressive obesity and DMBA exposure, potentially interfering with the role of CX37 during follicular development, maintenance of the germinal vesicle and ovulation.

Cx43 mRNA and protein levels were increased in lean ovaries after 18 wks which was similar to previous study that CX43 protein expression was increased in wild-type littermates compared to *Gja1*$^{Jrt/+}$ mutant mice after 11 wks of age in ovaries (Flenniken *et al.*, 2005). However, *Cx43* mRNA and protein levels were decreased in ovaries of obese mice, relative to their control littermates. CX43 forms channels between granulosa cells,

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which is required for their proliferation (Gittens, 2003). Granulosa cells recovered from *Cx43*$^{-/-}$ mice fail to show electrical coupling (Tong *et al.*, 2005). Thus, CX43 function is critical for ovarian function. A high fat diet induced low CX43 protein levels, which was restorable by pharmacological intervention in cardiac tissues of 32 wk old mice (Noyan-Ashraf *et al.*, 2013). Also, decreased CX43 levels in autopsied uterine tissue were demonstrated in rats fed a high fat and high cholesterol diet (Elmes *et al.*, 2011). Our data thus exhibit that CX43 protein levels are decreased during progressive obesity consistent with data in non-ovarian tissues.

DMBA exposure decreased *Cx43* mRNA and total protein levels both in lean and obese mice ovaries in agreement with our previous study in neonatal female rat pups ovaries which indicated that DMBA reduced *Cx43* mRNA and protein levels prior to follicle loss (Ganesan and Keating, 2014). Since DMBA is a component of cigarette smoke these results are in line with previous studies showing that cigarette smoke reduced CX43 expression in the corporal cavernosum of male rats (Liu *et al.*, 2011) and in human pancreatic ductal epithelial cells (Tai *et al.*, 2007). *Cx43* mRNA and protein were also decreased in atretic follicles in rat ovaries (Wiesen and Midgley, 1994). Further, an increase in apoptotic follicles associated with a decrease in the ovarian CX43 expression was shown in acute hyperglycemic and chronic diabetic female mice (Chang *et al.*, 2005). Immunohistochemistry staining in our study localized CX43 protein between the granulosa cells and CX43 protein staining intensity was reduced in obese ovaries. The intensity of CX43 protein staining was also reduced by DMBA exposure in lean and obese ovaries. The reduced amount of CX43 protein staining by DMBA exposure and by obesity supports that atresia is ongoing in antral follicles.

Cx45 mRNA and protein were decreased in both lean and obese ovaries after 12 wks of age but CX45 protein increased after 18 wks of age in lean ovaries. In contrast, the level of *Cx45* mRNA and protein were both reduced after 18 wks of age in obese relative to lean ovaries. Also, *Cx45* mRNA and total protein levels were reduced in obese ovaries compared to lean ovaries after DMBA exposure. CX45 is localized between granulosa cells and expressed throughout follicular development (Okuma *et al.*, 1996; Wright *et al.*, 2001). We show that CX45 protein is present between the granulosa cells of antral follicles and the intensity of protein staining was reduced by obesity. This is the first study to explore the level of ovarian CX45 protein after chemical exposure, and results indicate that CX45 is reduced by both DMBA exposure and progressive obesity. Additionally, CX45 is reported to co-localize with CX43 protein in rat ovaries (Okuma *et al.*, 1996). Our results suggest that both CX43 and CX45 protein staining intensity were reduced by obesity and that CX43 may be more sensitive to DMBA exposure than CX45.

Taken together, ovarian *Cx37*, *Cx43*, and *Cx45* show a dynamic pattern of expression and we provide evidence for decreased *Cx37*, *Cx43*, and *Cx45* as a consequence of ovarian aging as well as progressive obesity. Also, for all three CX's studied, obesity had an additive impact on DMBA exposure; CX37 and 43 were decreased while CX45 was increased, relative to their respective controls. From 12 weeks of age onwards, primordial and primary follicles were reduced by progressive obesity while secondary and antral follicle numbers were increased (Nteeba *et al.*, 2014b). Thus the decreased levels of CX's observed are

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potentially a reflection of reduced small pre-antral follicles that are present in the ovary. In the DMBA exposure study, although ovaries from obese females exposed to DMBA had lower ovarian weights, there was no apparent different in the number of follicles at any stage of development due to DMBA exposure (Nteeba *et al.*, 2014a). In that study, however, reduced numbers of small pre-antral follicles with increased numbers of larger follicle types were also observed due to obesity, thus, the decrease in ovarian CX37 and CX43 in the obese females exposed to DMBA could be due to changes in CX levels in non-follicular cells or attributable to loss of small pre-antral follicles. However, using immunofluorescence staining, we chose the same number of follicles from which to measure CX protein staining and found reduced staining, lending support that changes observed in total CX protein are likely to reflect alterations within follicles, rather than simply a decline in follicle number, although this cannot be completely ruled out by these studies. Interestingly, in somatic cells and paradoxical to our findings, increased levels of CX43 as well as gap junction communication during programmed cell death has been noted (Wilson *et al.*, 2000). This could represent an attempt by the tissue to protect against cell death, and our future studies are aimed at deciphering the impacts of both obesity and DMBA exposure on oocyte and granulosa *Cx* gene expression. In addition, determining whether ovarian impacts on *Cx* expression is consistent with those observed in non-ovarian tissues will be an important area for follow-on experiments.

In summary, we report that ovarian aging, obesity and DMBA exposure each alter connexin gap junction protein expression in ovaries in a manner that could contribute to compromised reproduction observed during each one of these physiological paradigms.

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Abbreviations

GJA4 or connexin 37; CX37	Gap junction protein alpha 4
GJA1 or connexin 43; CX43	Gap junction protein alpha 1
GJC1 or connexin 45	Gap junction protein gamma 1
DMBA	7,12-dimethylbenz[a]anthracene
TEMED	N′N′N′N′-Tetramethylethylenediamine
GLP-1	Glucagon-like peptide-1

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Highlights

- Ovarian gap junction proteins are affected by ovarian aging and obesity.
- DMBA exposure negatively impacts gap junction proteins.
- Altered gap junction proteins may contribute to infertility.

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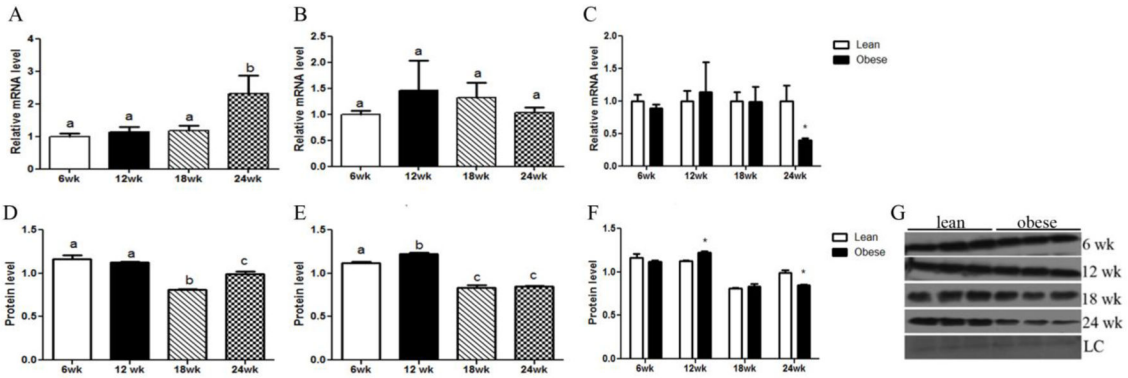

Figure 1. Effect of progressive obesity on *Cx37* mRNA and protein abundance

Ovaries were collected from 6, 12, 18 or 24 wks aged mice to isolate RNA and protein to perform qRT-PCR (A–C) and western blot (D–G). Analysis between lean alone (A, D), obese alone (B, E) and between lean and obese or interaction (C, F) and values are expressed as fold change ± SE for mRNA expression and raw data mean ± SE for protein expression; n=3. Different letters or asterisk (*) indicate the statistical significance at $P < 0.05$.

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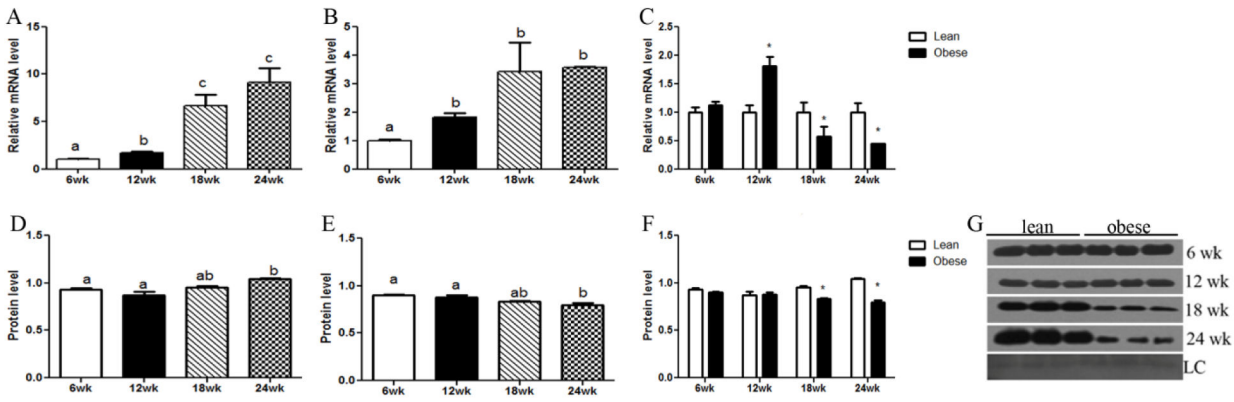

Figure 2. Progressive obesity impact on *Cx43* mRNA and protein level

Ovaries were collected from 6, 12, 18 and 24 wks of age to isolate RNA and protein to perform qRT-PCR (A–C) and western blot (D–G). Analysis between lean alone (A, D), obese alone (B, E) and between lean and obese or interaction (C, F) and values are expressed as fold change ± SE for mRNA expression and raw data mean ± SE for protein expression; n=3. Different letters or asterisk (*) indicate the statistical significance at $P < 0.05$.

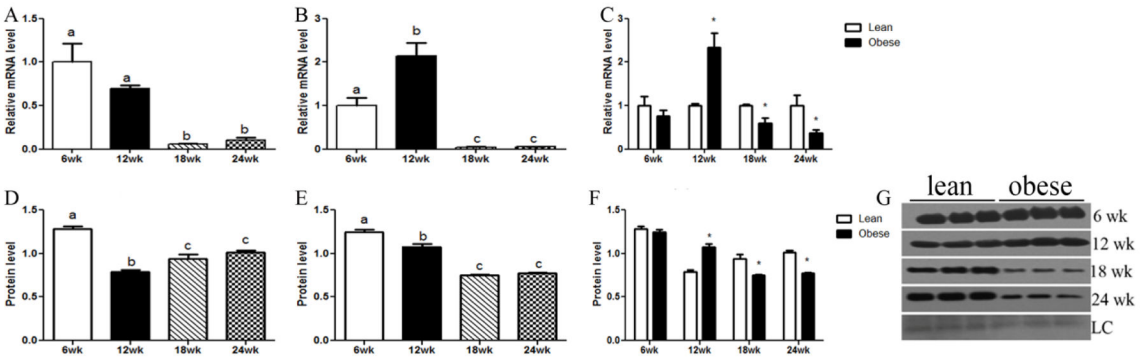

Figure 3. Effect of obesity on ovarian *Cx45* mRNA and protein abundance

Ovaries were collected from 6, 12, 18 and 24 wks of age to isolate RNA and protein to perform qRT-PCR (A–C) and western blot (D–G). Analysis between lean alone (A, D), obese alone (B, E) and between lean and obese interaction (C, F) and values are expressed as fold change ± SE for mRNA expression and raw data mean ± SE for protein expression; n=3. Different letters or asterisk (*) indicate statistical significance at $P < 0.05$.

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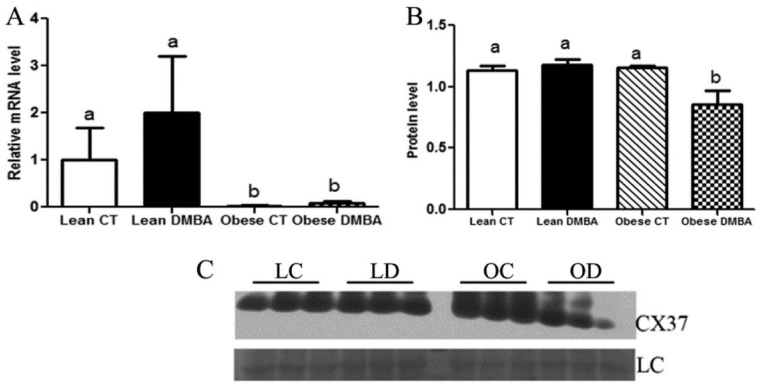

Figure 4. DMBA effects on *Cx37* mRNA and protein level

Following exposure to DMBA at 1mg/kg for 14 days, total mRNA and protein were isolated to perform qRT-PCR (A) and western blot analysis (B, C). Values are expressed as fold change ± SE for mRNA expression and raw data mean ± SE for total protein; n=3. Different letters indicate the statistical significance at $P < 0.05$.

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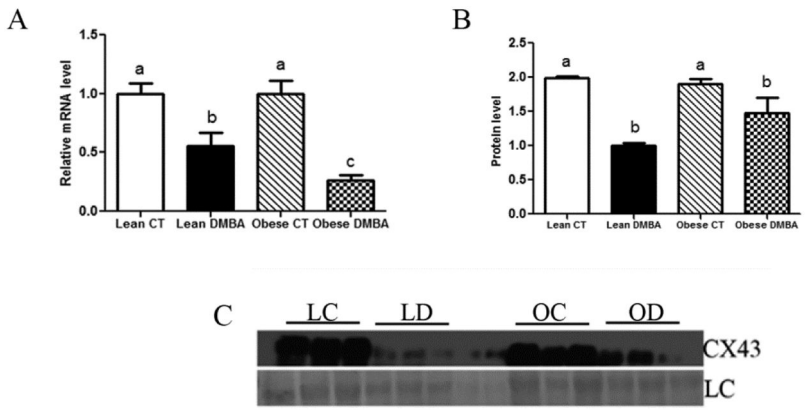

Figure 5. Impact of DMBA exposure on ovarian *Cx43* mRNA and protein

Following exposure of DMBA at 1mg/kg for 14 days, total mRNA and protein were isolated to perform qRT-PCR (A) and western blot analysis (B, C). Values are expressed as fold change ± SE for mRNA expression and raw data mean ± SE for total protein; n=3. Different letters indicate the statistical significance at $P < 0.05$.

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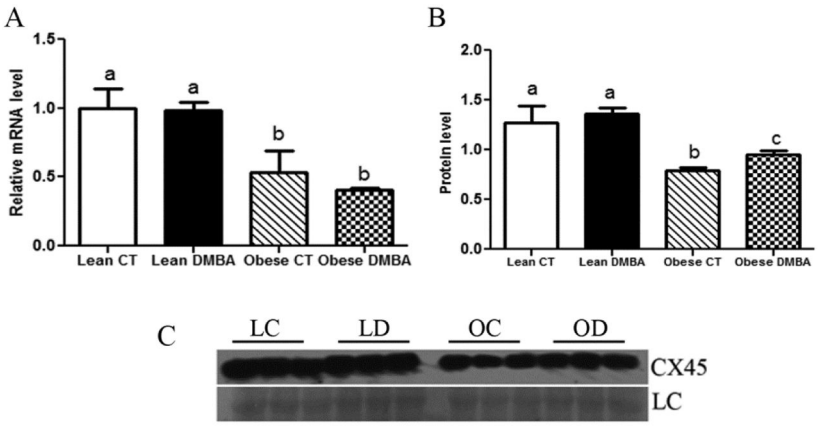

Figure 6. Consequence of DMBA exposure on *Cx45* mRNA and protein expression

Following exposure of DMBA at 1mg/kg for 14 days, total mRNA and protein were isolated to perform qRT-PCR (A) and western blot (B,C) analysis. Values are expressed as fold change ± SE for mRNA expression and raw data mean ± SE for total protein; n=3. Different letters indicate the statistical significance at $P < 0.05$.

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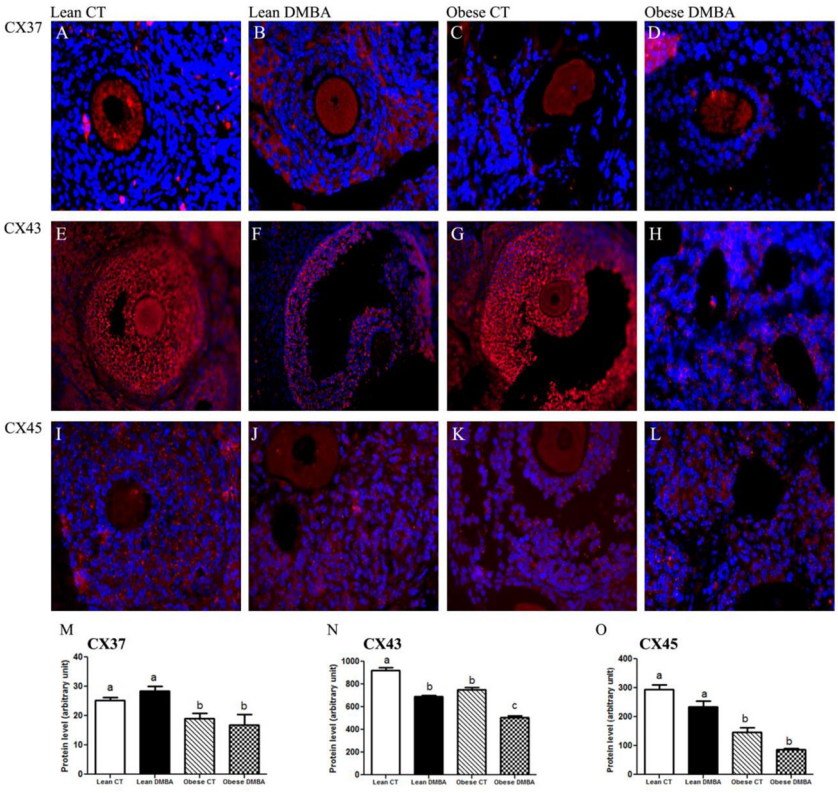

Figure 7. Localization and quantification of obesity and DMBA impacts on CX proteins

Following exposure of DMBA at 1mg/kg for 14 days, paraffin embedded ovarian sections were immunostained using primary CX37, CX43 and CX45 antibodies to check the localization (A–L) and intensity of staining for CX37 (M); CX43 (N) and CX45 (O). ImageJ software was used to analyze the intensity of staining (M–O). 10 large follicles per ovary and 3 ovaries were used for protein analysis. Values are expressed as mean ± SE. Different letters indicate the statistical significance at $P < 0.05$.

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Table 1

Primer sequence used for qPCR

Gene	Forward primer	Reverse primer
Cx37	TGATCACAGGTGGTTCTGGA	AGGAGAAGTGGGGTGTGATG
Cx43	TGCAAGTGTGTAAGCGTGTG	TTGCACGGCAGGAATTCTAT
Cx45	TGGTTGGGCTTAAAACTTGG	CAGCTCCACCTTCAGAGTCC
Gapdh	GTGGACCTCATGGCCTACAT	GGATGGAATTGTGAGGGAGA